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Diferulate Cross-Links Impede the Enzymatic Degradation of Non-Lignified Maize Walls

J H Grabber,* R D Hatfield and J Ralph

US Dairy Forage Research Center, USDA-Agricultural Research Service, Madison, Wisconsin 53706, USA

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Abstract: We assessed the effect of ferulate substitution and diferulate cross-linking of xylans on the degradation of cell walls by two fungal enzyme mixtures, one of which contained feruloyl esterase and high xylanase activities. Non-lignified cell suspensions of maize (*Zea mays*) were grown with 0 or 40 μM 2-aminoindan-2-phosphonic acid to produce walls with normal (17·2 mg g^{-1}) or reduced (5·1 mg g^{-1}) ferulate concentrations. Walls were incubated with mercaptoethanol to inhibit diferulate formation or with hydrogen peroxide to stimulate diferulate formation by wall bound peroxidases. Varying the ferulate substitution of xylans did not affect cell wall hydrolysis. In contrast, increasing ferulate dimerisation from 18 to 40% reduced carbohydrate release by 94–122 mg g^{-1} after 3 h and by 0–48 mg g^{-1} after 54 h of enzymatic hydrolysis. Diferulate cross-links impeded the release of xylans, cellulose and pectins from walls. These results provide compelling evidence that diferulate cross-links reduce the rate and, to a lesser degree, the extent of wall hydrolysis by fungal enzymes. Our results also suggest that enzyme mixtures containing high xylanase activity but not feruloyl esterase activity can partially overcome the inhibitory effects of diferulate cross-linking on wall hydrolysis. © 1998 SCI.

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Key words: *Zea mays*, Gramineae; ferulic acid; diferulic acids; dehydrodiferulic acids; xylan; cell wall; degradability; cellulase; xylanase; feruloyl esterase

INTRODUCTION

Ferulates are esterified to the C5-hydroxyl of α-L-arabinose sidechains on grass xylans (Kato and Nevins 1985; Mueller-Harvey *et al* 1986). Xylans are cross-linked by oxidative coupling of ferulate monomers into dehydrodimers (Ralph *et al* 1994; Grabber *et al* 1995). Ferulate substitution and diferulate cross-linking of xylans are thought to structurally impede the enzymatic degradation of grass walls (Hatfield 1993; Jung and Deetz 1993) but unambiguous evidence for this is lacking. Most approaches used to isolate or model the effects of ferulates on wall degradability have serious limitations. For example, random and non-specific chemical attachment of ferulate esters (and possibly polyesters) to xylans, cellulose and cell walls reduced polysaccharide degradability (Sawai *et al* 1983; Sahlu and Jung 1986; Jung *et al* 1991). However, this system poorly models the regiospecific acylation of xylans by ferulate that is observed in grasses (Bohn and Fales 1989; Jung *et al* 1991). Chemical hydrolysis of ester linkages improves wall degradability (Morrison 1991; Fritz *et al* 1991; Jung *et al* 1992) but these treatments are not specific enough to isolate the effects of ferulates on digestion (Fry 1986). In contrast, some enzymes cleave ferulate ester linkages with high specificity, but their activity on cell walls is extremely low (Coughlan and Hazlewood 1993).

Previous work by our group demonstrated that ferulate substitution and diferulate cross-linking of xylans are realistically modelled and readily manipulated in cell suspensions of maize (Grabber *et al* 1995). This

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system was used to determine whether ferulate substitution or diferulate cross-linking of xylans limits the degradation of nonlignified cell walls by crude fungal carbohydrases. A secondary objective was to determine whether a fungal enzyme mixture containing feruloyl esterase and high xylanase activity could lessen the impact of ferulates and diferulates on cell wall hydrolysis.

EXPERIMENTAL

Preparation, chemical composition and degradability of non-lignified walls

In the first experiment, non-lignified cell suspensions of maize (*Zea mays* cv. Black Mexican) were grown with 0–50 μM 2-aminoindan-2-phosphonic acid (AIP) to manipulate the deposition of ferulate esters into walls (Grabber *et al* 1995). After 16 days of culture (early-stationary growth phase), cells were suspended in ice-cold 25 mM HEPES buffer and 25 mM mercaptoethanol (pH 7·0) and ruptured by a probe-type sonicator. Cell walls were collected on a nylon mesh (20 μm) and washed sequentially with 1% SDS, water and acetone to remove cytoplasmic contaminants. Ester-linked ferulates and diferulates in walls were released by 2 M NaOH (16 h at 25°C), extracted into ether, and analysed by GLC (Ralph *et al* 1994; Grabber *et al* 1995). Cell walls were suspended (1%, w/v) in 20 mM acetate buffer (pH 4·8, 40°C) and degraded with a mixture of Viscozyme L and Celluclast 1.5 L, each added at 0·04 μl mg^{-1} of cell wall. After 4 and 48 h of hydrolysis, wall residues were pelleted by centrifugation (2500 × *g* for 10 min) and an aliquot of the supernatant was analysed for total carbohydrate (Dubois *et al* 1956). Data from two replicates were averaged and subjected to regression analysis to determine how carbohydrate release from walls was affected by variation in total ferulate and diferulate concentrations.

In a separate study, nonlignified walls from cell suspensions grown with 0 or 40 μM AIP were incubated with mercaptoethanol or with H_2O_2 as described previously (Grabber *et al* 1995). Treatments were replicated two times within a single experiment. Cell walls were analysed for neutral sugars (Hatfield and Weimer 1995), uronic acids (Blumenkrantz and Asboe-Hansen 1973) and ester-linked ferulates and diferulates (Ralph *et al* 1994; Grabber *et al* 1995). Cell walls were degraded with a mixture of Viscozyme and Celluclast as described earlier and with a mixture of Biofeed Beta (CT form, 0·04 mg mg^{-1} of cell wall) and Celluclast 1.5 L (0·04 μl mg^{-1} of cell wall) in 20 mM MES buffer (pH 6·0, 40°C). Periodically, wall residues were pelleted by centrifugation (2500 × *g* for 10 min) and an aliquot of the supernatant was analysed for total carbohydrate (Dubois *et al* 1956). Supernatants from Viscozyme and Celluclast digestions were also analysed for uronic acids (Blumenkrantz and Asboe-Hansen 1973) and for neutral sugars following TFA hydrolysis (Hatfield and Weimer 1995). A first-order kinetic model was used to describe the release of sugars from walls (Grabber *et al* 1992). Chemical composition and degradability data were analysed as a 2 × 2 factorial in a completely random design. Kinetic data were analysed as a completely random design in a split-plot arrangement with AIP and H_2O_2 treatments as whole plots and sugars as subplots (Steel and Torrie 1980).

Isolation and partial characterisation of hydrolase-resistant wall fractions

Cell walls from maize cell suspensions were isolated and treated with H_2O_2 according to methods described by Grabber *et al* (1995). Cell walls (2 g) were degraded for 72 h with Viscozyme and Celluclast as described above. The hydrolysate was centrifuged (2500 × *g* for 10 min) and the pellet was washed several times with water using centrifugation and finally freeze-dried to yield an indigestible residue fraction (218 mg). Supernatants were combined, filtered (1·2 μm retention) and freeze dried. Freeze-dried supernatant was dissolved in 40 ml of water and 160 ml of ethanol was added. After 16 h at −20°C, the precipitate was collected by centrifugation (10 000 × *g* for 30 min), redissolved in water, and reprecipitated by ethanol. After centrifugation, the pellet was freeze-dried to yield an oligosaccharide fraction (272 mg). Wall fractions were analysed for neutral sugars, uronic acids and ester-linked ferulates and diferulates. Acetylation of the oligosaccharide fraction was checked by ^{13}C-NMR. The linkage structure of the oligosaccharide fraction was determined by methylation analysis (Carpita and Shea 1989).

Assays for feruloyl esterase activity

Ferulic acid esterase activity in the enzyme preparations was assayed using methyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (FA-Ara) as a substrate (Hatfield *et al* 1991). One unit of activity (1 U) was defined as the amount of enzyme releasing 1 μmol of free ferulic acid min^{-1} at pH 6·0 and 40°C. Feruloyl esterase activity was also evaluated by incubating the indigestible residue and oligosaccharide fractions for 24 h with the Biofeed and Celluclast mixture described above. After centrifugation (2500 × *g* for 10 min), an aliquot of the supernatant was analysed for total carbohydrate (Dubois *et al* 1956) and reducing sugars (Nelson 1944; Somogyi 1952). The hydrolysate was then acidified (pH < 2) with HCl and extracted immediately with ether to isolate ferulates released by esterases. Ferulates

were analysed by GC-FID using 2-hydroxycinnamic acid as an internal standard (Ralph *et al* 1994; Grabber *et al* 1995).

RESULTS AND DISCUSSION

Manipulation of ferulate deposition and cross-linking in non-lignified maize walls

Cell walls from maize cell suspensions contained 195 mg g^{-1} of arabinose, 172 mg g^{-1} of xylose, 78 mg g^{-1} of galactose, 312 mg g^{-1} of glucose, 8 mg g^{-1} of rhamnose, 112 mg g^{-1} of uronic acids, 18 mg g^{-1} of total ferulates (ferulates plus diferulates), and 0·4 mg g^{-1} of *p*-coumarate. If 90% of the xylosyl residues were derived from xylan (Carpita 1984), then about 1 in every 11 xylosyl residues of the xylan backbone were substituted with feruloylated arabinose. The walls also contained about 100 mg g^{-1} of protein (Kieliszewski and Lamport 1988), a portion of which are peroxidases capable of coupling ferulate monomers into dehydrodimers when H_2O_2 is present (Grabber *et al* 1995). Walls from these suspensions are essentially nonlignified, containing only trace amounts of ferulate ethers and 3 mg g^{-1} of guaiacyl lignin (Grabber *et al* 1995, 1996). Overall, the composition of cell walls are representative of primary walls of grasses (Darvil *et al* 1978; Carpita 1984).

Ferulate ester deposition into cell walls was reduced up to 75% by growing cell suspensions in the presence of AIP, a specific inhibitor of phenylalanine ammonia lyase (Fig 1). The proportion of diferulates to total ferulates increased when suspensions were treated with AIP, suggesting that cells responded to reduced feruloylation

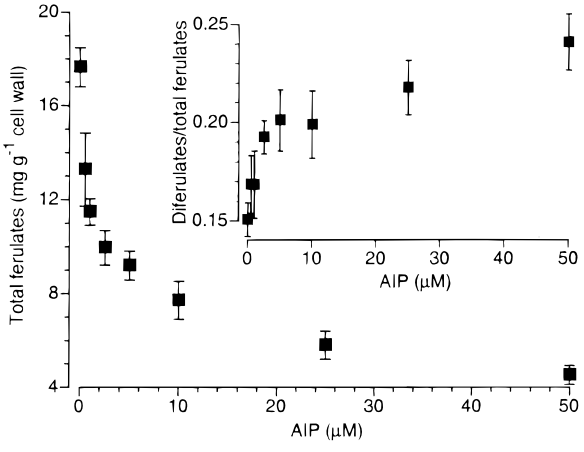

Fig 1. Total ferulate concentrations (monomers plus dimers) in cell walls isolated from maize cell suspensions grown in the presence of 0–50 μM AIP. The inset shows how AIP treatment increased the ratio of diferulates to total ferulates in cell walls. Data points represent the means (with standard error bars) of two replications.

of xylans by increasing the extent of diferulate cross-linking. Previously we found that AIP treatment of maize suspensions had minor effects on the carbohydrate composition of walls (Grabber *et al* 1995). Other studies indicate that structural protein and cellulose deposition are not affected by AIP treatment (Keller *et al* 1990; Schmutz *et al* 1993). In addition to reducing ferulate concentrations, AIP treatment of maize cell suspensions reduced the concentrations of other minor phenolic components (lignin and *p*-coumarate) in cell walls (Grabber *et al* 1995).

Hydrogen peroxide treatment of non-lignified walls with normal or low feruloylation increased the proportion of diferulates to total ferulates from *c* 18 to 44% without affecting the total ferulate concentration in walls (Table 1). The carbohydrate composition of walls was not affected by H_2O_2 treatment. Overall, AIP and H_2O_2 treatments provide a relatively specific means of manipulating the ferulate substitution and diferulate cross-linking of cell walls.

Effect of ferulate substitution and diferulate cross-linking on the degradability of non-lignified maize walls

Hydrolysis with Viscozyme and Celluclast

Cell wall degradability was first assessed with an equal mixture of Viscozyme L and Celluclast 1.5 L. Viscozyme, from *Aspergillus* sp, is marketed as a mixed-linked β-glucanase preparation that also contains pectinase, cellulase and xylanase activities. In a recent study, Viscozyme, in combination with an α-amylase, provided good estimates of the *in situ* rumen fermentable organic matter of a variety of feedstuffs (Cone *et al* 1996). Celluclast is a crude cellulase from *Trichoderma reesei* that also contains significant xylanase, mixed-linked β-glucanase, and protease activities (Massiot *et al* 1989). Celluclast readily degrades cellulosic substrates and its cellulase complex has been thoroughly characterised (Kolev *et al* 1991; Yu and Saddler 1995; Micard *et al* 1997). Our assay with FA-Ara revealed that both of these enzyme preparations were free of feruloyl esterase activity.

Weinberg *et al* (1990, 1995) found that an equal mixture of Viscozyme and Celluclast was particularly effective for degrading cell walls and this was confirmed by our work with non-lignified maize walls. Uronic acids, galactose and glucose residues were released from maize walls more rapidly than arabinose and xylose residues, but all sugars were released to a similar extent (Fig 2). Using greater quantities of the enzyme mixture increased the rate but not the extent of cell wall hydrolysis. A mixture of these preparations gave a two-fold greater rate of wall hydrolysis than a comparable volume of either preparation used alone, confirming that Viscozyme and Celluclast had complementary enzyme activities (data not shown). Based on these

TABLE 1

Ferulate concentrations and degradability of structural carbohydrates (SC) in non-lignified maize walls ($n = 2$). Feruloylation of walls was manipulated by growing cell suspensions with and without AIP, a specific inhibitor of phenylalanine ammonia lyase. Peroxidase-mediated coupling of ferulate monomers into dimers was limited by isolating and incubating walls with mercapoethanol or stimulated by incubating walls with H_2O_2. Walls were hydrolysed with a mixture of Viscozyme and Celluclast (VC) or Biofeed and Celluclast (BC).

AIP (μM)	*H_2O_2* (*mmol*)	*Ferulates* (*mg g^{-1} cell wall*)			*Carbohydrate released*					
					VC				*BC*	
		Monomers	*Dimers*	*Total*	*3 h* (*mg g^{-1} SC*)	*54 h* (*mg g^{-1} SC*)	*Rate* (h^{-1})	*Extent* (*mg g^{-1} SC*)	*3 h* (*mg g^{-1} SC*)	*54 h* (*mg g^{-1} SC*)
Normal feruloylation										
0	0	14·53	2·62	17·15	357	856	0·176	862	546	871
0	0·4	8·96	6·65	15·61	243	794	0·108	820	416	856
Low feruloylation										
40	0	3·75	1·31	5·06	460	898	0·244	916	570	898
40	0·4	2·27	2·25	4·52	329	865	0·136	900	511	916
Analysis of variance[a]										
AIP		*	*	*	*	*	*	*	*	*
H_2O_2		*	*	NS	*	*	*	*	*	NS
AIP × H_2O_2		*	*	NS	NS	NS	NS	*	NS	NS

[a] *, NS, significant at the 0·05 level of probability and not significant, respectively.

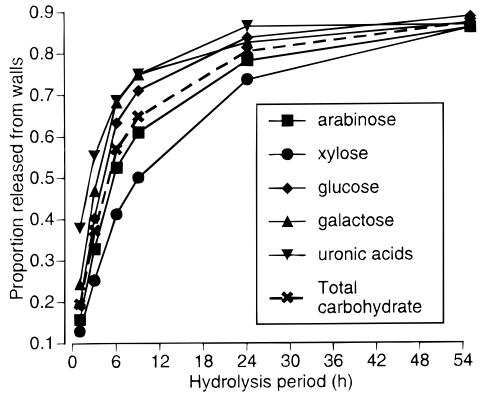

Fig 2. Release of sugars from non-lignified maize walls hydrolysed with a mixture of Viscozyme and Celluclast. Data points represent the means of two replications.

results, we concluded that this enzyme mixture was suitable for evaluating how ferulate substitution and diferulate cross-linking affects the degradability of non-lignified maize walls.

The quantity of carbohydrate released from walls by Viscozyme and Celluclast was increased by AIP treatment (Fig 3 and Table 1), indicating that ferulate substitution and/or diferulate cross-linking limited cell wall degradability. As mentioned earlier, AIP treatment also reduced the concentration of minor phenolic components (eg lignin and *p*-coumarate esters) and slightly altered the carbohydrate composition of walls, so it is likely that degradability was also enhanced somewhat by wall modifications not directly related to ferulate substitution or diferulate cross-linking.

In contrast to the AIP treatment, H_2O_2 treatment provided a highly specific means of assessing whether diferulate cross-links limit the degradability of non-lignified walls. When walls with normal or low feruloylation were treated with H_2O_2, peroxidase-mediated coupling of ferulate monomers into dehydrodimers reduced total carbohydrate release by 122 mg g^{-1} after 3 h of hydrolysis; the rate of carbohydrate release was reduced by 42% (Table 1). H_2O_2 treatment reduced the rate at which all neutral and acidic sugars were released from walls (Table 2), suggesting that diferulate cross-linking of xylans reduces the accessibility of hydrolytic enzymes to all structural polysaccharides in walls. In contrast, variation in diferulate cross-linking had comparatively little effect on the extent of carbohydrate release (measured at 54 h or estimated by nonlinear regression, Table 1). The extent of arabinose and xylose release, indicative of xylan degradation, was reduced by the highest level diferulate cross-linking (Table 3). Variation in cross-linking did not effect the extent to which cellulosic and pectic sugars were released from walls. Regardless of the degree of ferulate substitution or diferulate cross-linking, monomers comprised *c* 50% of the arabinose, 60% of the galactose and 90% of the glucose released from walls. In contrast, monomers comprised *c* 10% of the xylose released from walls, suggesting that solubilised xylans were poorly degraded by the enzyme mixture.

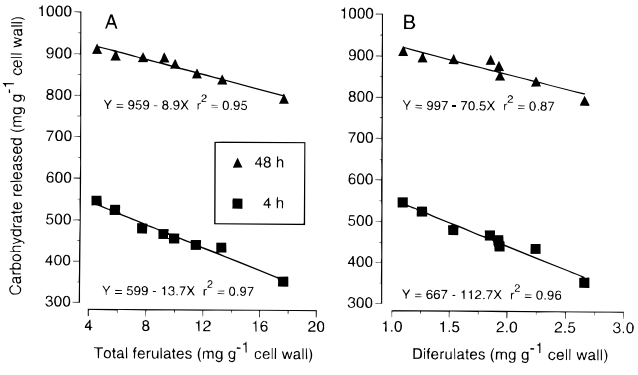

Fig 3. Relationships between hydrolase degradability and ferulate concentrations in non-lignified walls isolated from maize cell suspensions grown with 0–50 μM AIP: (A) total ferulates (monomers plus dimers), and (B) diferulates. Cell walls were hydrolysed with Viscozyme and Celluclast. Data represent the means of two replications.

The role of ferulates and diferulates in limiting xylan degradation was investigated in greater detail by isolating and partially characterising oligosaccharides and indigestible residues remaining after hydrolysis of H_2O_2 treated walls. Ethanol precipitation yielded an oligosaccharide fraction representing 15% of the dry matter, 26% of the ferulate, and 62% of the diferulate released into the hydrolysate (Table 4). The fraction contained 11·4 mg g^{-1} of ferulates, 24·8 mg g^{-1} of diferulates, 247 mg g^{-1} of arabinose, 331 mg g^{-1} of xylose, 134 mg g^{-1} of uronic acids, 68 mg g^{-1} of galactose, 37 mg g^{-1} of rhamnose, and 10 mg g^{-1} of glucose. Acetyl groups were not detected by ^{13}C-NMR. Based on monosaccharide and methylation analyses, xylo-oligosaccharides comprised about 60% of the carbohydrate in this fraction; the balance consisted of oligosaccharides derived from rhamnogalacturonans and arabinogalactans. The backbone of the xylo-oligosaccharides had an average DP of 4·25 and about 50% of the xylose residues were substituted with terminal arabinose. Based on the total ferulate and xylose content of the fraction, we estimate that only about one in 12 xylose units in the backbone were substituted with feruloylated arabinose. Therefore, it appears that only a portion of the xylo-oligosaccharides were substituted with ferulates or cross-linked by diferulates. This suggests that ferulates were not the major factor limiting hydrolysis of these xylo-oligosaccharides. Rather, the structure of the xylo-oligosaccharide indicates that its degradation was limited by inadequate α-L-arabinofuranosidase and β-D-xylosidase activities in the enzyme mixture. Indigestible residues contained 11% of the dry matter, 35% of the ferulates and 40% of the

TABLE 2
Rate of sugar release from non-lignified maize walls hydrolysed with Viscozyme and Celluclast as affected by AIP treatment of cell suspensions and H_2O_2 treatment of isolated cell walls ($n = 2$)

AIP (μM)	H_2O_2 (*mmol*)	*Ferulates* (*mg g^{-1} cell wall*)		*Arabinose*[a]	*Xylose*[a]	*Glucose*[a]	*Galactose*[a]	*Uronic acids*[a]
		Dimers	*Total*					
Normal feruloylation								
0	0	2·62	17·15	0·154	0·098	0·209	0·269	0·303
0	0·4	6·65	15·61	0·084	0·055	0·112	0·137	0·209
Low feruloylation								
40	0	1·31	5·06	0·219	0·156	0·249	0·377	0·371
40	0·4	2·25	4·52	0·113	0·062	0·152	0·213	0·250

[a] Rate constant (h^{-1}). LSD = 0·036. LSD to compare means within columns ($P = 0·05$).

diferulates present in the original wall. This fraction contained 29·1 mg g^{-1} of ferulates, 32·8 mg g^{-1} of diferulates, 214 mg g^{-1} of arabinose, 296 mg g^{-1} of xylose, 62 mg g^{-1} of galactose, 57 mg g^{-1} of glucose and 34 mg g^{-1} of uronic acids. Except for lower concentrations of pectic sugars, the carbohydrate composition of indigestible residues was quite similar to that of the xylo-oligosaccharide fraction. About one in 6 xylose residues were substituted by arabinose acylated with ferulate or diferulate, a substitution rate about 80% greater than that estimated for the original wall. Since these highly feruloylated xylans accumulated after H_2O_2 treatment, it is apparent that diferulate cross-linking prevented their hydrolysis.

Hydrolysis with Biofeed and Celluclast

The effects of ferulate substitution and cross-linking on cell wall degradability were also evaluated with a

TABLE 3
Potential extent of sugar release from non-lignified maize walls hydrolysed with Viscozyme and Celluclast as affected by AIP treatment of cell suspensions and H_2O_2 treatment of isolated cell walls ($n = 2$)

AIP (μM)	H_2O_2 (*mmol*)	*Ferulates* (*mg g^{-1} cell wall*)		*Arabinose*[a]	*Xylose*[a]	*Glucose*[a]	*Galactose*[a]	*Uronic acids*[a]
		Dimers	*Total*					
Normal feruloylation								
0	0	2·62	17·15	0·851	0·868	0·873	0·853	0·852
0	0·4	6·65	15·61	0·785	0·687	0·839	0·830	0·856
Low feruloylation								
40	0	1·31	5·06	0·910	0·937	0·904	0·940	0·880
40	0·4	2·25	4·52	0·893	0·914	0·929	0·906	0·866

[a] Proportion of sugar released. LSD = 0·040. LSD to compare means within columns ($P = 0·05$).

TABLE 4
Ferulate and diferulate composition (mg g^{-1}) of non-lignified walls and wall fractions recovered after a 72 h incubation with Viscozyme and Celluclast. Values in parentheses indicate the percentage of each constituent released as free acids from wall fractions after a 24 h incubation with Biofeed and Celluclast. Data represent the means of duplicate analyses

	(*Z*)-*Ferulate*	(*E*)-*Ferulate*	(*E*)-*Diferulates*			
			8–8	*8–5*	*8–O–4*	*5–5*
Cell wall	2·51	6·64	1·05	5·17	1·53	1·24
Solublised oligosaccharides	1·69 (0)	9·74 (83)	3·08 (0)	13·83 (12)	3·65 (0)	4·27 (24)
Indigestible residues	7·21 (0)	21·90 (52)	3·22 (0)	17·92 (8)	6·00 (0)	5·66 (13)

mixture of Celluclast 1.5 L and Biofeed Beta. Biofeed is marketed as xylanase, mixed β-glucanase and amylase preparation from *Humicula insolens* and *Bacillus amyloliquefaciens*. We found that the Biofeed preparation also contained 15 mU of feruloyl esterase activity per mg of solid using FA-Ara as a substrate. The feruloyl esterase was extremely stable; incubation for 96 h resulted in no loss of activity. Feruloyl esterase activity was also evaluated with the oligosaccharide and indigestible residue fractions described earlier. These fractions were hydrolysed with a Biofeed and Celluclast mixture containing 0·6 mU of feruloyl esterase activity per mg of substrate, theoretically enough activity to completely release all ferulates within 5–9 h of incubation. A 24 h incubation released 71% of the ferulates and only 11% of the diferulates contained in the oligosaccharide fraction (Table 4). Similar treatment of indigestible residues released only 39% of the ferulates and 7% of the diferulates contained in this fraction. The feruloyl esterases exhibited a high degree of substrate specificity, releasing only (*E*)-ferulate and (*E*)-diferulates coupled by 5–5 and 8–5 linkages. Although several types of 8–5 coupled diferulates are released from walls by saponification (Ralph *et al* 1994), only the decarboxylated form was released by the feruloyl esterase. In addition to releasing ferulates, Biofeed and Celluclast released 168 mg g^{-1} of reducing sugars from the oligosaccharide fraction and 737 mg g^{-1} of total carbohydrate from the indigestible residue fraction, indicating that this enzyme mixture had greater xylanase activity than the Viscozyme and Celluclast mixture. Subsequent work has shown that Biofeed and Celluclast degrades xylans at a two-fold greater rate than Viscozyme and Celluclast, whereas the degradation of cellulose was similar for both enzyme mixtures (Grabber J H unpublished).

It is well established that feruloyl esterases act synergistically with other xylanolytic enzymes to degrade soluble xylans; their activity on insoluble substrates is extremely poor (Coughlan and Hazelwood 1993). This was confirmed in a recent study where purified feruloyl esterases from *Aspergillus niger* and *Pseudomonas fluorescens* released only a small amount (1–9%) of ferulates and essentially no diferulates (0–0·7%) from barley and wheat cell walls. Adding xylanase dramatically improved the release of ferulate but not diferulates by the esterases (Bartolome *et al* 1997). The addition of a purified feruloyl esterase from *Aspergillus awamori* to Celluclast enzymes also had no effect on hydrolysis of cell walls from Italian ryegrass (McCrae *et al* 1994). Therefore, it appears that feruloyl esterase activity will not affect the degradation of cell walls by fungal carbohydrases.

Although feruloyl esterase activity was not expected to affect degradability, we hydrolysed cell walls with Biofeed and Celluclast to investigate whether the inhibitory effects of diferulate cross-linking could be mitigated by an enzyme mixture containing high xylanase activity. As noted earlier with Viscozyme and Celluclast, concurrent reductions in ferulate substitution and diferulate cross-linking, due to AIP treatment, increased the quantity of carbohydrate released with Biofeed and Celluclast (Table 1). H_2O_2/peroxidase-mediated coupling of ferulate monomers into dehydrodimers reduced carbohydrate release by 94 mg g^{-1} after 3 h of hydrolysis; differences were not significant after 54 h of hydrolysis. Although diferulate cross-linking reduced the initial hydrolysis of walls by both enzyme mixtures, degradation was more rapid and extensive with Biofeed and Celluclast, particularly for walls with high levels of diferulate cross-linking. These results indicate that high xylanse activity can enhance the degradation of highly cross-linked walls, however, additional work with purified xylanases is needed to confirm these preliminary observations.

It is also of interest to note that walls with similar diferulate concentrations (2·62 vs 2·25 mg g^{-1}) but substantially different total ferulate concentrations (17·15 vs 4·52 mg g^{-1}) had roughly the same degradability (Tables 1, 2 and 3). This suggests that ferulate substitution of xylans did not affect the rate or extent of cell wall hydrolysis.

CONCLUSIONS

Our results provide compelling evidence that the rate, and to a lesser degree, the extent of wall degradation is restricted by diferulate cross-linking of xylans. In contrast, simple feruloylation of xylans does not appear to impede cell wall hydrolysis. The inhibitory effects of diferulate cross-linking may be partially alleviated if a hydrolase preparation contains high xylanase activity. In a future paper, we will report how ferulate–lignin cross-linking affects the hydrolysis synthetically lignified maize walls.

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